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IDENTIFICATION AND QUANTIFICATION OF PINE NEEDLE ESSENTIAL OIL FROM DIFFERENT HABITATS AND SPECIES OF CHINA BY GC-MS AND GC METHOD

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Abstract

Background: Fresh pine needles, which evergreen, renewable and can be collected at any time, have abundant essential oil. The pine needle essential oil possess various biological activities, including antimicrobial, antioxidant, anti-inflammatory, cardioprotective properties *etc*., and can be widely used as medicine or medical raw materials, fragrances *etc.* So in order to make full use of pine needle plant resources, especially essential oil, the identification and quantification of essential oil were investigated.
Materials and Methods: GC-MS was used to identified compounds in pine needle essential oil of *Pinus massoniana* Lamb. from Zhejiang, and GC method was developed for determining 5 compounds (namely α-pinene, β-pinene, limonene, bornyl acetate, β-caryophyllene) in pine needle essential oil from different habitats of *Pinus massoniana* Lamb. and different species (*Pinus koraiensis* Sieb. et Zucc., *Pinus sylvestris* var. mongolica Litv., *Abies holophylla* Maxim.). Hierarchical clustering analysis (HCA) was used to evaluate homogeneity of pine needles in China.
Results: 42 compounds(monoterpenes and sesquiterpenes) were identified by GC-MS, which accounted for 99.62% of total essential oil, particularly contained α-and β-pinene (45.23%). Quantification results showed content of bornyl acetate were the lowest, while content of α-pinene, β-pinene were all high except Sichuan, among them α-pinene were higher than β-pinene except for Shandong and Jiangsu, and total content of essential oil in Guangxi is the highest, Jiangsu was the lowest within *Pinus massoniana* from different habitats. Within pine needle from different species, the highest total content was *Pinus koraiensis*, the lowest was *Pinus sylvestris*, Interestingly, the highest content of bornyl acetate was *Abies holophylla*. By HCA, samples were sorted into two clusters, *Abies holophylla* and the other, that is *Abies holophylla* is different from *Pinus massoniana*, *Pinus koraiensis* and *Pinus sylvestris*. *Pinus massoniana* from Sichuan is different from other Pinus, in which limonene was the highest in all samples.
Conclusions: Compounds of pine needle essential oil vary greatly between genus, while smaller between species, so composition differences among pine needle essential oil was related with genus and species. *Abies holophylla* is different from other pinus species, and *Pinus massoniana* from Sichuan is different from other habitats. Pine needle essential oil contains abundant α-and β-pinene, which can be used as an alternative raw materials source of pinene. And *Abies holophylla* and *Pinus massoniana* from Sichuan can become bornyl acetate and limonene alternative source of raw materials.

Keywords: Pine needle; Essential oil; Terpene; GC-MS; GC

List of Abbreviations: GC: Gas chromatography; **GC-MS:** Gas chromatography coupled with mass spectrometry detector; **FID:** flame ionization detector; **HCA:** Hierarchical clustering analysis; **RSD:** relative standard deviations.

Introduction

Pine needle is the leaves of Pinaceae, such as *Pinus massoniana*, *Pinus koraiensis*, *Pinus sylvestris* var., *Abies holophylla* Maxim., *Pinus tabulaeformis*, *etc*, which is one of the worldwide distribution plant, especially in China. Pine needle was first recorded in "Compendium of Materia Medica", and is widely used in China for treatment of rheumatism and joint pain(Raynal, 2007, Cisar et al., 2008), swelling and pain from injuries, night blindness, hypertension, neurasthenia and external use in treating chilblain(Meng et al., 2012, Suntar et al., 2012, Clark et al., 2014). Phytochemical studies showed abundant compounds presented in pine needle, such an essential oil, flavonoids, Shikimic acid, amino acids, lignin(Kim et al., 2010, Kwon et al.,2010, Zhang and Wang, 2010), among which essential oil were considered to be the primary bioactive constituents. Essential oil content is higher in pine needle, has a special fragrance, mainly contains monoterpene, sesquiterpene and a small amount of terpene alcohols, esters, such as α-pinene, β-pinene, limonene, β-caryophyllene, etc (Nikolic et al., 2007; Zeng et al., 2012; Satyal, Paudel et al. ,2013). The pine needle essential oil were reported to possess various biological activities, involving antimicrobial (Lee et al., 2009, Amri et al., 2011; Politeo et al., 2011), antifungall (Satyal et al., 2013), antioxidant (Kwak et al., 2006; Park and Lee, 2011; Emami et al., 2013; Chaudhary et al., 2014), anti-inflammatory (Suntar et al., 2012), anti-tussive and expectorant, hypolipidemic (Kim et al., 2012; Ko et al., 2013), anti-hypertension, anti-diabetic (Joo et al., 2013), cardioprotective properties, anticancer (Wei et al., 2008; Jo et al., 2012; Yousuf Dar., 2012) etc.

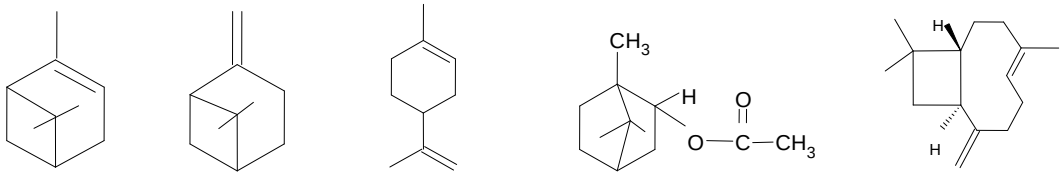
Most pine trees which are evergreen, sustainable renewable are considered a source of bioactive compounds which have achieved important contributions to the discovery of pharmaceutical materials and other biomedical applications. And several bioactive compounds have been isolated from different Pinus species that have various biological effects. Moreover, pine needle essential oil is widely used as medicine or medical raw materials, fragrances in cosmetics, flavoring additives for food and beverages, scenting agents in a variety of household products(Macchioni et al., 2002), and intermediates in the synthesis of perfume chemicals (Comhaire and Mahmoud, 2003; Draelos, 2011). To increase our understanding of the pharmacological activities of pine needle and disclose the secret underlying its efficacy, it is essential to make further comprehensive study of active constituents in pine needle. Although numerous studies have been carried out to characterize the essential oil compounds and content in pine needle, rather limited investigations have been conducted into the chemical profile in pine needle from different habitats and species.

In this paper, GC-MS and GC method were used for identification and quantitative of pine needle essential oil respectively. The 42 compounds were identified; the main compounds were α-pinene, β-caryophyllene, β-pinene, and limonene, Bornyl acetate etc. The above five compounds from pine needle essential oil of different habitats and different species were determined. And hierarchical clustering analysis (HCA) was used to evaluate homogeneity of pine needles in China.

Materials and Methods

Chemicals and Reagents

α-pinene(97%), β-pinene(99%), limonene(95%), caryophyllene(98.5%) (Figure 1) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Bornyl acetate (100%), was purchased from National Institutes for Food and Drug Control (Beijing, China). N-Hexane and anhydrous sodium sulfate (all analytically pure) were purchased from China Petrochemical Group Hangzhou Refinery (Hangzhou, China), Hangzhou Chemical Reagents Co. Ltd. (Zhejiang, China) and Shanghai No.4 Reagent & H.v Chemical Co., Ltd. (Shanghai, China) respectively. Deionized water was prepared by using Milli-Q system (Millipore, Bedford, MA, USA).



α-pinene(2) β-pinene(4) Limonene(6) Bornyl acetate(10) β-Caryophyllene (17)

Figure 1: Chemical structures of investigated compounds

Plant

The pine needles of *Pinus massoniana* Lamb. were collected from different habitats of China, and *Pinus koraiensis* Sieb. et Zucc., *Pinus sylvestris* var. mongolica Litv., *Abies holophylla* Maxim. were collected from Changbai Mountain of Jilin province (Table 4). These pine needles were identified and confirmed by the Pro. K.R. Chen, Zhejiang Chinese Medical University (China).

Sample Preparation

Samples were cut into 1-2cm pieces. Fresh plant material (200g) were immersed in deionized water (1200mL) and submitted to steam distillation using an apparatus with condenser. The essential oil was extracted by hydrodistillation

for 3hrs. The obtained essential oil was separated, dried (anh. Na_2SO_4), and stored at 4℃in a sealed vial for further use. The essential oil diluted to 5 mL with n-hexane prepared before being used.

Qualitative GC-MS Analysis

An Agilent 6890 gas chromatograph equipped with an Agilent 5973 MSD (Agilent Technologies, Palo Alto, CA, USA) were used for GC-MS analysis. Gas chromatographic separation was carried out with a HP-5 capillary column (30m×0.25 mmI.D, film thickness: 0.25μm). Sample (0.2μL) was injected manually in split mode (spilt ratio was 90:1). The injector temperature was 290℃ and the helium carrier gas flow-rate was 1.0 mL/min. The oven temperature was programmed 50℃ (hold 2min) raised to 100 ℃(hold 2 min) at 5 ℃/min, then to 290 ℃(hold 7 min) at 20℃/min. MSD ion source was electron ionization, and the temperature was 280℃. All mass spectra were recorded at 70 eV.

The essential oil compounds of *Pinus massoniana* Lamb. from Zhejiang were identified by comparing their retention time and the mass spectra with those of the NIST 02 mass spectral library (National Institute of Standards and Technology) provided by the software of GC-MS system, and previously published literature or standard control. The relative content of the essential oil compounds were calculated using area normalization method.

GC Analysis

An Agilent 6890 gas chromatograph equipped with 7683B series injector and FID detector were used for GC analysis. Gas chromatographic separation was carried out with a HP-5 capillary column (30m×0.25mmI.D, film thickness: 0.25μm). Sample (1μL) was injected automatically in split mode (spilt ratio was 50:1). The pressure was 47kPa, injector temperature was 230℃, detector temperature was 250℃ and the nitrogen carrier gas flow-rate was 1.0mL/min. The oven temperature was programmed 50℃(hold 10min) raised to 120℃ at 2℃/min, then to 250 ℃(hold 10 min) at 10℃/min.

Method Validation

Calibration Curves

N-hexane stock solutions containing 5 reference compounds were prepared and diluted to a series of appropriate concentrations with n-hexane, and injected into GC for analysis. The calibration curves were constructed by plotting the peak areas versus the amount of each analyte.

Precision, Repeatability, Accuracy and Stability

For the precision of the developed method, the prepared samples were analyzed for six replicates within one day, and calculated relative standard deviations (RSD) of peak areas. The repeatability of the developed method was operated as "sample preparation" for six replicates, and expressed with RSD of content. The retention stability of the developed method was evaluated with peak areas and the RSD of peak areas at 0 h, 3 h, 6 h, 9 h, 12 h, 15 h and 24h. The recovery was performed by adding a known amount of individual standards into a certain amount (100g) of pine needle sample. The mixture was extracted and analyzed using the method mentioned above. Six replicates were performed for the test. All used samples in the developed method were *Pinus massoniana* Lamb. from Zhejiang.

Data Analysis

Hierarchical clustering analysis(HCA) was performed by SPSS 19.0 for windows (SPSS Inc., Chicago, IL, USA), which comprise a number of "procedures"-graphical, statistical, reporting, processing and tabulating procedures-that enable simple and rapid data evaluation. HCA is an exploratory data analysis tool used for quantifying similarities among samples. Its purpose is to sort samples into clusters so that the degree of association is strong between members of the same cluster and weak between members of different clusters. The relationship of each cluster can be visualized in a dendrogram. HCA was used to examine the possible grouping of samples.

Results and Discussion

Qualitative Analysis and Identification of Chemical

45 compounds were detected and 42 compounds were identified by GC-MS of *Pinus massoniana* Lamb. from Zhejiang, accounted for 99.62% of total essential oil. The identified 42 compounds were summarized in Table 1, meanwhile listed retention times, quality and relative content. And the representative GC-MS TIC chromatogram were shown in Figure1.The main compoundsidentified were monoterpenes (59.57%, including 12 compounds) and sesquiterpenes (40.04%, including 30 compounds), particularly contained **α-pinene**(35.18%), **β-caryophyllene**(15.50%), **β-pinene** (10.05%), **limonene**(4.34%), δ-cadinene (3.58%), germacrene D(3.18%), γ-elemen(2.76%),α-caryophyllene(2.74%), and **bornyl acetate**(2.31%), camphene (2.14%), β-elemen(1.94%), terpinolene(1.77%) *etc.*

Table 1: Chemical compounds of pine needle essential oil

NO.	t_R(min)	Compounds	Qual%	contents %
1	4.31	Tricyclene	97	0.21
2	**4.61**	**α-pinene**[a]	**94**	**35.18**
3	**4.82**	**Camphene**	**97**	**2.14**
4	**5.43**	**β-pinene**[a]	**94**	**10.05**
5	5.81	β-Myrcene	62	0.73
6	**6.65**	**limonene**[a]	**53**	**4.34**
7	**8.18**	**Terpinolene**	**96**	**1.77**
8	10.94	α-Terpineol	96	0.97
9	12.81	Linalyl acetate	90	1.12
10	**13.63**	**Bornyl acetate**[a]	**97**	**2.31**
11	15.04	1,5,5-Trimethyl-6-methylene-cyclohexene	81	0.20
12	15.32	(+)-4-Carene	94	0.54
13	15.71	Copaene	98	0.18
14	15.85	Levo-b-elemene	60	0.15
15	**15.97**	**β-Elemen**	**58**	**1.94**
16	16.07	(+)- Longifolene	99	0.14
17	**16.33**	**β-Caryophyllene**[a]	**99**	**15.50**
18	16.42	β-Cubebene	98	0.08
19	16.52	Eremophilene	70	0.36
20	**16.69**	**α-Caryophyllene**	**98**	**2.74**
21	16.78	(Z)- β-Farnesene	97	0.85
22	16.83	Bicyclo[7.2.0]undec-4-ene,4,11,11-trimethyl-8-methylene	86	0.09
23	**16.98**	**Germacrene D**	**93**	**3.18**
24	17.03	(-)-α-Selinene	99	0.62
25	17.09	Naphthalene,1,2,3,4,4a,5,6,8a-octahydro-7-ethyl-4-methylene-1-(1-methylethyl)-,(1.alpha.,4a.beta.,8a.alpha.)	87	0.11
26	**17.14**	**γ-Elemen**	**87**	**2.76**
27	17.18	α-Muurolene	99	0.59
28	17.30	Naphthalene,1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)	98	1.13
29	**17.40**	**δ-Cadinene**	**95**	**3.58**
30	17.48	Cadina-1,4-diene	97	0.07
31	17.51	(-)-α-Cadinene	96	0.13
32	17.58	α-Calacorene	78	0.10
33	17.78	Peruviol,d-Nerolidol	80	0.25
34	17.87	Caryophyllene oxide	86	1.29
35	17.92	(-)-Globulol	76	0.22
36	17.98	(+)-γ-Gurjunene	92	0.22
37	18.09	cis-Z-α-Bisabolene epoxide	86	0.17
38	18.14	(-)-γ-Cadinene	79	0.09
39	18.24	Naphthalene,1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)	96	0.17
40	18.33	tau-Cadinol	99	1.55
41	18.44	α-Cadinol	96	1.64
42	19.58	farnesyl alcohol	91	0.15
Compound class				
Monoterpenes				59.57
Sesquiterpenes				40.04
Total				99.62

[a] Confirmed by standard compounds

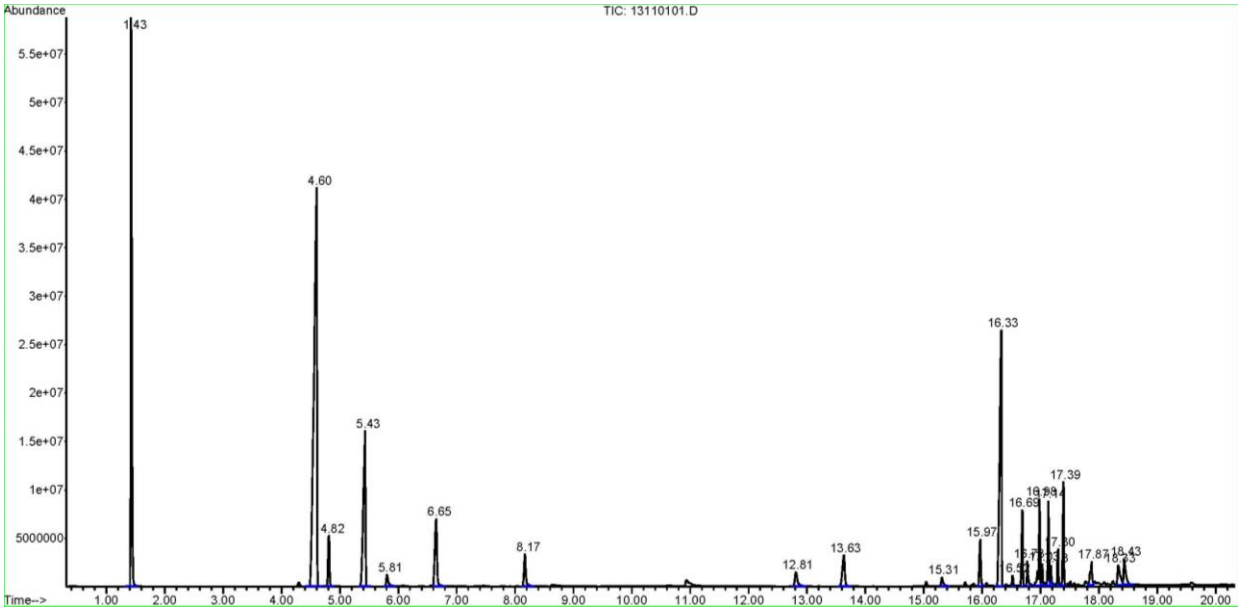


Figure 2: GC-MS TIC chromatogram of pine needle essential oil

Optimized Extraction Procedure of Essential Oil

The optimization of extraction procedure was performed using pine needle of *Pinus massoniana* Lamb. from Zhejiang. The parameters, including immersion time (0 h,1 h,2 h,3 h and 4 h), extraction time (3 h,5 h,7 h,9 h and 11 h) and solvent dosage (4 times,5 times,6 times and 8 times), were studied by using univariate approach. The total amount of 5 investigated compounds was used as marker for evaluation of extraction efficiency. The exhausted extraction procedure was determined by the total amount of 5 investigated compounds. Figure 2 showed that the extraction efficiency of essential oil decreased with immersion time and extraction time extended. And the best solvent dosage was 6 times of sample. Finally, the optimized method proposed were as follows: solvent, water; no immersion time; extraction time, 3 h; solvent dosage, 6 times of sample.

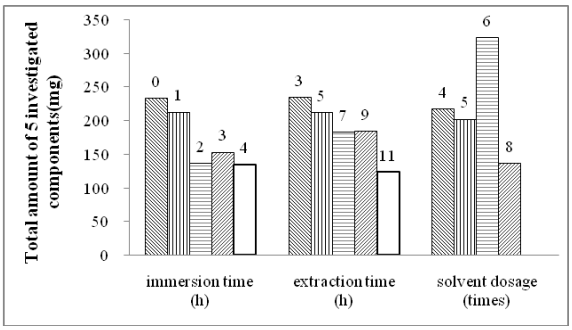


Figure 3: Effects of immersion time, extraction time and solvent dosage of 5 investigated compounds of pine needle essential oil

Method Validation

The linearity, regression, and linear ranges of 5 analytes were determined using the developed GC method. The data indicated a good relationship between the investigated compounds' amount and their peak areas within the test ranges (R^2 >0.9997). The Regressive equation, test range and correlation coefficient were shown in Table 2. And the precision RSD of 5 analytes was less than 1.35%, repeatability RSD was less than 4.53%, retention stability in 24 h was less than 3.80%. The results were shown in Table 2.

Accuracy is expressed by the recovery rate. The recoveries were between 98.1% and 101.0% (shown in Table 3). These results showed that the developed GC method was sensitive, precise and accurate for quantitative determination of 5 investigated compounds.

Table 2: Linear regression data and precision of 5 investigated compounds

Analytes	Linear regression data			Precision (RSD, %, n=6)	Repeatability (RSD, %, n=6)	Stability (RSD, %, n=7)
	Regressive equation	Test range (μg)	R^2			
2	Y=182.53X-18.434	0.671~33.552	1.0000	1.27	4.53	3.80
4	Y=177.8X-32.796	0.696~34.806	0.9998	1.10	4.14	3.78
6	Y=183.09X-42.909	0.466~9.312	0.9998	0.61	4.31	3.77
10	Y=139.87X-9.8881	0.704~7.036	0.9998	0.76	3.75	3.47
17	Y=192.35X-13.026	0.367~18.353	0.9997	1.35	3.50	3.66

Table 3: Recoveries for the assay of 5 investigated compounds in *Pinus massoniana* Lamb.

Analytes	Original(mg)	Added(mg)	Found [a](mg)	Recovery [b](%)	RSD(%)
2	54.23	105.22	158.02	98.7	1.78
4	21.32	39.29	60.41	99.5	3.19
6	7.69	13.12	20.84	100.3	3.24
10	2.76	6.31	9.14	101.0	1.04
17	18.88	37.27	55.43	98.1	1.65

[a] The data were present as average of six determinations.
[b] Recovery(%)=100×(found amount−original amount)
spike amount .

Quantification of Investigated Compounds in Pine Needle Essential Oil

Compounds in pine needle were well separated using the developed GC method. Typical chromatograms from different areas of China were shown in Figure 3, and the content of 5 investigated compounds were summarized in Table 4 and Figure 4. GC determination found that content of α-pinene were high in all pine needles, content of limonene, β-pinene and bornyl acetate were rich in *Pinus massoniana* Lamb. from Sichuan, *Pinus koraiensis* Sieb. et Zucc., *Abies holophylla* Maxim. respectively, and the highest total content of 5 investigated compounds was *Pinus massoniana* Lamb. from Guangxi. The results showed that content in different habitats and different species were variant greatly.

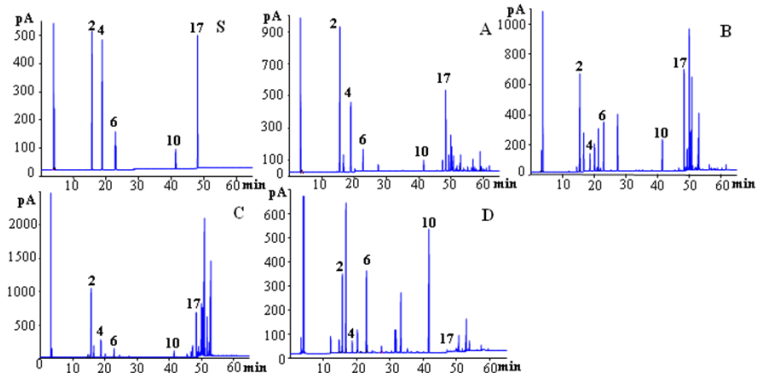


Figure 4: GC chromatograms of: (S) mixed standard, (A) *Pinus massoniana* Lamb. of Zhejiang Lin'an, (B) *Pinus koraiensis* Sieb. et Zucc., (C) *Pinus sylvestris* var. mongolica Litv., (D) *Abies holophylla* Maxim.

Table 4: Content of 5 investigated compounds in pine needle essential oil

Sample code	Species	Collection site	The rate of essential oil (%,V/W)	Investigated compounds(%, g/ml)					Total (%, g/mL)
				2	4	6	10	17	
A	*Pinus massoniana* Lamb.	Zhejiang Lin'an	0.35	20.77	9.87	3.52	2.02	7.17	43.36
B		Hubei Suizhou	0.54	28.93	10.38	3.75	2.87	6.61	52.55
C		Guangxi Heshan	0.44	25.02	13.07	5.34	3.02	9.97	56.42
D		Hunan Hengyang	0.64	26.54	8.17	4.38	2.22	10.60	51.92
E		Henan Pingdingshan	0.35	26.03	2.23	8.13	0.31	5.27	41.96
F		Shandong Linyi	0.34	8.55	21.40	5.14	1.06	5.45	41.60
G		Yunnan Dali	0.41	11.83	5.91	8.06	1.54	6.69	34.03
H		Sichuan Yibin	0.14	7.26	2.07	22.70	-	9.44	41.48
I		Jiangsu Zhenjiang	0.23	7.98	15.92	1.20	-	4.73	29.84
J	*Pinus koraiensis* Sieb. et Zucc.	Jilin Changbai Mountain	0.51	11.00	23.23	5.96	4.50	6.11	50.79

K	*Pinus sylvestris* var. mongolica Litv.	0.80	12.30	2.80	2.53	2.51	3.98	24.12
L	*Abies holophylla* Maxim.	0.35	7.55	1.38	8.58	20.09	0.14	37.74

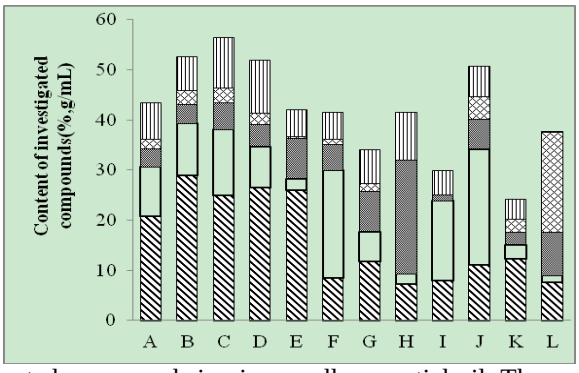


Figure 5: Comparison of 5 investigated compounds in pine needle essential oil. The compounds number (▨) 2, (□)4, (■) 6, (▩) 10, (▥) 17 were the same as in Table1. The sample codes were the same as in Table 4.

Hierarchical Clustering Analysis (HCA)

HCA of 12 tested pine needle essential oil (including 9 samples of *Pinus massoniana* Lamb. and 3 other species) was performed using 5 investigated compounds and 12 selected peaks(peak No.2, 3, 4, 6, 7, 10, 15, 17, 20, 23, 26,29, were bold in Table 1) as markers respectively (shown in Figure 5). Their results were very similar, which sort all samples into two clusters, *Abies holophylla* Maxim. and the other. The results showed that, the differences of essential oil between *Abies holophylla* Maxim. and the other pine needle were large, which in accordance with the fact that *Pinus massoniana* Lamb., *Pinus koraiensis* Sieb. et Zucc., *Pinus sylvestris* var. mongolica Litv. belong to Pinus, while *Abies holophylla* Maxim. belongs to Abies.

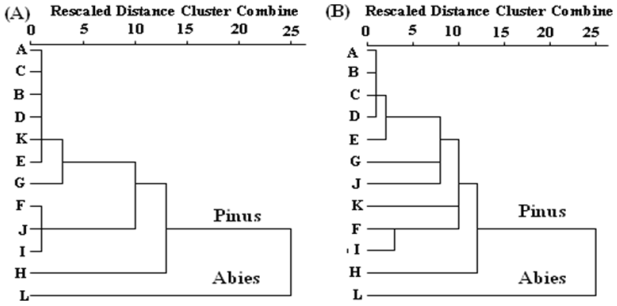


Figure 6: Dendrograms of HCA for 12 tested essential oil(including 9 samples of *Pinus massoniana* Lamb. and 3 other species)based on(A)5 investigated compounds and (B)12 marker peaks derived from their GC profile(be bold in Table 1). The sample codes were the same as in table 4.

Conclusion

Compounds of pine needle essential oil vary greatly between genus, while smaller between species, so composition differences among pine needle essential oil was related with genus and species. This paper reminder that *Abies holophylla* Maxim. is different from other pinus species, and *Pinus massoniana* Lamb. from Sichuan is different from other habitats.

Pine needle essential oil contains abundant α-pinene and β-pinene, which can replace pinene raw material as a sustainable renewable resource. The content of limonene, bornyl acetate were rich in *Pinus massoniana* Lamb. from Sichuan, *Abies holophylla* Maxim. respectively, so alternative resource of bornyl acetate can choose *Abies holophylla* Maxim., limonene can choose *Pinus massoniana* Lamb. from Sichuan.

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Declaration: Authors declare that there is no conflict of interest.

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